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ANNOTATED BIBLIOGRAPHY OF SUBTILIN
Assay, Biological Production, Purification and Chemistry, Biological Activity

J. C. Lewis

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ANNOTATED BIBLIOGRAPHY OF SUBTILIN

J. C. Lewis

The genus Bacillus has a long record of observed antibiotic activity. This bibliography covers reports of the antibiotic activity produced by a particular strain of Bacillus subtilis first studied from this aspect in the Western Regional Research Laboratory, and of the antibacterial agent, subtilin, concentrated from cultures of this organism. This strain of B. subtilis was originally obtained from N. R. Smith of the U. S. Department of Agriculture, Bureau of Plant Industry, Soils, and Agricultural Engineering, under the number 231. It is now deposited in the American Type Culture Collection as number 6633 and in the culture collection of the Northern Regional Research Laboratory as B-543.

Those articles originating from the Western Regional Research Laboratory are marked "WRRL" following the authors' names; those from the Medical School of the University of California, San Francisco 22, California, are marked "UC"; and those from the Department of Bacteriology, University of California at Los Angeles, California, are marked "UCLA".

Assay

Lewis, J. C., Humphreys, E. M., Thompson, P. A., Dimick, K. P., Benedict, R. G., Langlykke, A. F., Lightbody, H. D., WRRL (authors Benedict and Langlykke, NRRL). The microbiological assay of subtilin. Arch. Biochem. 14(3):437-450. 1947. Subtilin was assayed by a turbidimetric method which depended on growth inhibition of Micrococcus conglomeratus, Streptococcus faecalis, or Staphylococcus aureus. The cultures were incubated for 4 or 5 hours at 37°C. under nonsterile conditions. The antibiotic activity of crude culture extracts of B. subtilis and of partially purified lots of subtilin could not be accounted for entirely in terms of a single active substance. Sixty to 70 percent ethanol was optimal for extraction of subtilin from crude cultures. The extraction was essentially complete in 15 minutes. Crude cultures lost substantial amounts of activity on steaming for 1 to 2 hours; the rate of destruction was faster at pH 6.4 to 7.1 than at 2.5.

Microbiological Production

Humfeld, H., and Feustel, I. C., WRRL. Utilization of asparagus juice in microbiological culture media. Proc. Soc. Expt. Biol. and Med. 54(2): 232-235. 1943. B. subtilis cultures grown for 24 hours at 35°C. on asparagus butt juice medium were adjusted to pH 2.5 and sterilized for 10 minutes at 10 lbs. of steam pressure. These preparations possessed marked antibiotic activity against Staphylococcus aureus, Micrococcus conglomeratus, Lactobacillus casei, and the plant pathogen, Phytomonas michiganensis. Less activity was found against the Gram-negative plant pathogens Phytomonas juglandis and Erwinia amylovora. (Reprints exhausted.)

Jansen, E. F., and Hirschmann, D. J., WRRL. Subtilin--An antibacterial product of Bacillus subtilis. Culturing conditions and properties. Arch. Biochem. 4(3):297-309. 1944. B. subtilis was grown in surface cultures on media containing sucrose, mineral salts, and several organic nitrogen sources including asparagine, glutaric acid, aspartic acid, casein, and tryptone. The addition of manganese (1 ppm.) proved essential for high antibiotic activity. Wide variations were noted in qualitative serial dilution tests of antibacterial activity against *Staphylococcus aureus*, *Micrococcus conglomeratus*, and *Lactobacillus casei* of cultures acidified to pH 2.5 and sterilized for 10 minutes at 10 lbs. of steam pressure. The material having antibacterial activity was named *subtilin*, but was believed to consist of more than one substance. A crude concentrate was prepared by centrifuging and vacuum-drying cultures adjusted to pH 4.7. The active material was reported to be soluble in aqueous ethanol but not in 95 percent ethanol, to be diffusible, to be heat-labile under alkaline conditions but relatively stable at pH 2.5, and to be inactivated by light and by formaldehyde. (Reprints exhausted.)

Anonymous WRRL (announcements and brief abstracts of work done at the WRRL on production, purification and assay of subtilin):
Production, concentration, properties, and assay of the antibiotic, subtilin. Mimeographed Circular AIC-106, Bureau of Agricultural and Industrial Chemistry, U. S. Department of Agriculture. 1946. A summary of work on subtilin done at the Western Regional Research Laboratory prior to 1946.
Production of subtilin--a new antibiotic. Drug and Cosmetic Industry 60, 478-479. 1947.
Subtilin output and purification. Oil, Paint, and Drug Reporter 151, 5, 55. 1947.
Subtilin from asparagus waste. Chem. and Engin. News 25, 1070-1071. 1947.

Lewis, J. C., Feeney, R. E., Garibaldi, J. A., Michener, H. D., Hirschmann, D. J., Traufler, D. H., Langlykke, A. F., Lightbody, H. D., Stubbs, J. J., and Humfeld, H., WRRL (authors Traufler and Langlykke, NRRL). Subtilin production in surface cultures. Arch. Biochem. 14(3):415-425. 1947. The highest yields of subtilin in shallow-layer cultures (as determined by the antibiotic activity of aqueous ethanol extracts) were obtained with a beet molasses medium, which required the addition of $(NH_4)_2HPO_4$ (0.8 percent) and manganese (50 ppm.). Good yields were obtained on media prepared from asparagus butt waste press juice, molasses and grain worts, and corn steep liquor. An incubator temperature of 35°C., with peak temperatures in the cultures 4° to 8° C. higher, was approximately optimal. Maximum yields were usually attained in cultures 1 to 2 cm. deep after 24 to 48 hours. Prolonged incubation of asparagus and beet molasses media resulted in a drop in antibiotic activity. The distribution of subtilin between pellicle and culture filtrate varied widely for different media. Two other strains of *B. subtilis* did not produce appreciable amounts of subtilin.

Stubbs, J. J., Feeney, R. E., Lewis, J. C., Feustel, I. C., Lightbody, H. D., and Garibaldi, J. A., WRRL. Subtilin production in submerged culture. Arch. Biochem. 14(3):427-435. 1947. Excellent yields of subtilin were produced in fermenters of one-liter capacity equipped with high-speed stirrers for dispersion of air. Asparagus-juice concentrate, when diluted to 8 percent solids, steamed for 30 minutes, adjusted to pH 7.0, inoculated and incubated at 35°C. under aeration at a rate of one volume of air per minute for 10 hours, gave the highest yields. Subtilin was also produced in similar yield but at a slower rate on media prepared from beet molasses.

Feeney, R. E., Humphreys, E. M., Lightbody, H. D., and Garibaldi, J. A., WRRL. Nutritional studies on the formation of subtilin by Bacillus subtilis in surface cultures (abstract). Fed. Amer. Socs. Expt. Biol. Proc. 6(1):250. 1947. Preliminary announcement of results presented in the 3 following papers. Moderate growth and antibiotic yields were obtained after 60 to 72 hours of incubation in shallow-layer cultures of the following synthetic medium (in percentage): sucrose, 10; asparagine, 0.2; glutamic acid, 0.2; citric acid, 0.05; Na_2SO_4, 0.4; $(NH_4)_2HPO_4$, 0.8; NaCl, 0.03, and traces of other salts. Requirements for potassium, magnesium, manganese, iron, and zinc were demonstrated. Calcium exerted a markedly deleterious effect.

Feeney, R. E., Lightbody, H. D., and Garibaldi, J. A., WRRL. Zinc as an essential element for growth and subtilin formation by Bacillus subtilis. Arch. Biochem. 15(1):13-17. 1947. Zinc was found essential for growth and subtilin formation by B. subtilis in shallow-layer stationary cultures. A zinc-free medium was prepared (by extraction with diphenylthiocarbazone in carbon tetrachloride) which permitted only slight growth and subtilin yields of less than 20 mg. per liter. This medium gave a linearly increasing response in subtilin yields when 0.03 to 0.5-1.0 ppm. of zinc was added. Maximum yields were obtained over the range of 1 to 10 ppm. of added zinc. A partial zinc deficiency was also observed on an unpurified medium. This appears to be the first demonstration of an absolute requirement for zinc in bacterial growth. Cadmium, the only element capable of substitution for zinc, was only partially effective.

Feeney, R. E., Garibaldi, J. A., and Humphreys, E. M., WRRL. Nutritional studies on subtilin formation by Bacillus subtilis. Arch. Biochem. (in press). The nutritional requirements for the production of relatively high levels of subtilin in shallow-layer cultures were restricted to an appropriate source of carbohydrate or related compound, inorganic sources of N, P, and S, and mineral elements. NH_3 and NO_3 were equally satisfactory, while organic sources of N were not superior to inorganic sources. Approximate quantitative requirements were: sucrose, 5 to 7 percent; N, 2000 ppm., P, 150 ppm., and S, 80 ppm.

Feeney, R. E., and Garibaldi, J. A., WRRL. Studies on the mineral nutrition of the subtilin-producing strain of Bacillus subtilis. Arch. Biochem. (in press). The mineral requirements required to produce 10 g. of dry cells per liter of medium containing sucrose, ammonium nitrogen, asparagine, and glutamic acid were approximately as follows (in ppm.): K, 125; Mg, 2.5; Fe, 1.2; Mn, 0.7; and Zn, 0.5. The requirements for subtilin formation were generally higher than the growth requirements. Rb replaced K for growth but not for subtilin production. Except for this and a partial replacement of Zn by Cd, the requirements for the above elements were specific. Ca had a detrimental effect on subtilin production, while citrate generally had a beneficial effect.

Purification and Chemistry

Dimick, K. P., Alderton, G., Lewis, J. C., Lightbody, H. D., and Fevold, H. L., WRRL. Purification and some properties of subtilin. Arch. Biochem. 15(1):1-11. 1947. Subtilin was obtained by extraction with 70 percent ethanol from pellicles produced by B. subtilis on asparagus butt juice medium. The extract was concentrated _in vacuo_ to remove ethanol, whereupon the active material precipitated. To remove inactive contaminants the dried filter cake was extracted successively with 95 percent ethanol, and with 85 percent ethanol containing 1 percent each of acetic acid and NaCl. The active material was then obtained in solution by extracting the residue with 0.16 _M_ acetate at pH 4.6. The extracts were treated with filtering aid (Hyflo Super-Cel)1/, deionized with exchange resins, concentrated, and lyophilized to yield a dull white powder. The subtilin preparation was soluble to more than 10 percent in salt-free acidified water, but to less than 0.5 percent at pH 6 to 9. In the presence of 0.5 percent NaCl at acid pH the solubility was less than 0.5 percent. The preparation was soluble in 0 to 80 percent ethanol, and in methanol, but not in dry ethanol, butanol, amyl alcohol, acetone, ether, petroleum ether, or chloroform. It was about 0.5 percent soluble in _n_-butanol saturated with water. It diffused fairly rapidly through Cellophane. The preparation contained 14 percent total (Kjeldahl) nitrogen. The amino nitrogen content of 1.6 percent increased to about 11 percent after acid hydrolysis. It contained 4.2 percent sulfur and a trace of phosphorus. It was levorotatory. Subtilin was inactivated by alkali, and the activity decreased on incubation with pepsin and trypsin. Dry subtilin preparations lost activity when stored for a period of months at room temperature, but dilute solutions at pH 2.5 appeared stable when stored in the refrigerator.

Dimick, K. P., Alderton, G., Lightbody, H. D., and Fevold, H. L., WRRL. A method for purification of subtilin (abstract). Fed. Amer. Socs. Expt. Biol. Proc. 6(1):247-248. 1947. Preliminary announcement of the method described in the following paper. Surface or submerged cultures of _B. subtilis_, adjusted to pH 2.5 with HCl, were extracted with 1/2 to 1 volume of _n_-butanol. The two phases were separated in a Sharples1/ centrifuge and 1/2 volume of petroleum ether was added to the aqueous butanol extract. The butanol-petroleum ether mixture was extracted 3 times with 1/10 volume of 1 percent acetic acid. Sodium chloride was added to the combined aqueous extracts to 6 percent concentration. A precipitate separated on the surface and was skimmed off. It was washed with petroleum ether and the excess salt solution was removed by filtration. The filter cake was dried by lyophilization and extracted with 95 percent alcohol (5 or 6 times with 20 ml. per gram) and subsequently in a similar manner with 85 percent ethyl alcohol containing 1 percent NaCl and 1 percent acetic acid. The extracts were discarded. The alcohol-insoluble residue was dissolved to a concentration of 1 percent in distilled water. The pH was adjusted to 4.5 with dilute NaOH and 0.4 percent NaCl added. The precipitate was removed by centrifugation, redissolved, and reprecipitated as before. This was repeated three or four times. The active extracts were deionized by ion-exchange resins and dried by lyophilization, or the active material was precipitated with 10 percent NaCl and subsequently dissolved, deionized, and dried.

1/ The mention of this product does not imply that it is endorsed or recommended by the Department of Agriculture over others of a similar nature not mentioned.

Fevold, H. L., Dimick, K. P., and Klose, A. A., WRRL. Isolation of subtilin from submerged cultures. Arch. Biochem. (in press). A method for the isolation of subtilin is described which depends on the extraction of the culture broth at pH 2 with one-half volume of n-butanol, addition of one-half volume of petroleum ether to the butanol extract and extraction with 1 percent acetic acid, precipitation from the aqueous extract with 10 percent NaCl, extraction of the moist cake with absolute ethanol, fractionation of a 0.5-0.75 percent solution of the cake with 0.4 percent NaCl at pH 4.6, reprecipitation of the soluble fraction with 10 percent NaCl, removal of excess NaCl by washing with 1 percent NaCl and then by absolute ethanol, and drying. About 50 percent over-all yields were obtained.

The product appeared to be essentially homogeneous by fractional dialysis, salt fractionation, and electrophoretic studies. The LD_{50} for subcutaneous injection in mice was about 3 gm. per kg. of body weight.

Lineweaver, H., Klose, A. A., and Alderton, G., WRRL. An alternate step for the isolation of subtilin. Arch. Biochem. 16(2):311-313. 1948. Subtilin may be salted out of butanol extracts of B. subtilis culture by adding 60 gm. of NaCl per liter of butanol and adjusting the pH of the aqueous phase to 5. The precipitate may be collected in the bowl of a Sharples continuous centrifuge which may be adjusted to remove separately the dehydrated butanol and the salt-water. Relatively little subtilin is lost in these liquid phases.

Lewis, J. C., and Jansen, E. F., WRRL. Enhancement of subtilin activity by methylation (abstract). Fed. Amer. Socs. Expt. Biol. Proc. 6(1):270. 1947. The bacteriostatic activity of subtilin against Micrococcus conglomeratus, Staphylococcus aureus, and Streptococcus faecalis was increased approximately 4-, 2-, and 5-fold, respectively, by treatment of the subtilin with dilute HCl in methanol. The greatest enhancement was found after 6 to 20 hours of incubation at room temperature of a 1 percent solution in 0.6 N HCl-90 percent methanol or after 20 to 40 hours in a 0.03 N HCl-absolute methanol. The isolated product contained 2.0 percent $-OCH_3$ by Zeisel as compared with a negligible test (0.15 percent) in the unmodified subtilin. The activity decreased markedly on more prolonged or more drastic treatment. Treatment of subtilin with diazomethane resulted in inactivation.

Stansly, P. G., and Ananenko, N. H., American Cyanamid Co. Resistance of polymixin to some proteolytic enzymes. Arch. Biochem. 15(3):473-474. 1947. Unlike polymixin, subtilin was largely inactivated by pepsin (pH 2.2-4.8), trypsin (pH 4.4-7.5), pancreatin (pH 4.4-7.5), and erepsin (pH 6.1-7.8).

Biological Activity

Salle, A. J., and Jann, G. J., UCLA. Subtilin--An antibiotic produced by Bacillus subtilis. I. Action on various organisms. Proc. Soc. Expt. Biol. and Med. 60, 60-64. 1945. Partially purified subtilin was found to inhibit growth of a number of Gram-positive bacteria, the Gram-negative Neisseria catarrhalis and N. gonorrhoeae, certain acid-fast bacteria including Mycobacterium tuberculosis, and certain pathogenic fungi. The effect on M. tuberculosis was bacteriostatic at low and bactericidal at higher concentrations.

Salle, A. J., and Jann, G. J., UCLA. Subtilin--Antibiotic produced by *Bacillus subtilis*. II. Toxicity of subtilin to living embryonic tissue. Proc. Soc. Expt. Biol. and Med. 61, 23-24. 1946. Partially purified subtilin possessed a low toxicity for embryonic chick heart tissue cultures. Exposure to a 0.2 percent solution for 10 minutes at 37° C. was required to kill the cultures. A concentration of 0.01 percent was required to kill *Staphylococcus aureus* under similar conditions.

Salle, A. J., and Jann, G. J., UCLA. Subtilin--Antibiotic produced by *Bacillus subtilis*. III. Effect on type III pneumococcus in mice. Proc. Soc. Expt. Biol. and Med. 62, 40-42. 1946. Partially purified subtilin in repeated doses of 0.05 mg. injected intraperitoneally quickly cured experimental type III pneumococcus infections induced in mice by intraperitoneal inoculation, without apparent toxic reaction in the animals. A group untreated until 9 hours after infection were in very bad condition before treatment, but appeared almost normal after 2 or 3 injections.

Salle, A. J., and Jann, G. J., UCLA. Subtilin--Antibiotic produced by *Bacillus subtilis*. IV. Effect of subtilin on the course of experimental anthrax infections in guinea pigs. Proc. Soc. Expt. Biol. and Med. 63, 41-42. 1946. Partially purified subtilin in doses of 6 mg. injected intraperitoneally at frequent intervals over a period of 11 days protected guinea pigs from experimental anthrax infections induced by intraperitoneal inoculation, without apparent toxic reaction in the animals. Initial treatments were made 3, 6, and 9 hours after inoculation. Untreated controls died in about 3 days.

Salle, A. J., and Jann, G. J., UCLA. Subtilin--Antibiotic produced by *Bacillus subtilis*. V. Effect on *Streptococcus pyogenes* infections in mice. Proc. Soc. Expt. Biol. and Med. 63, 519-520. 1946. Partially purified subtilin in 0.1 or 0.2 mg. repeated doses injected intraperitoneally quickly cured experimental *Streptococcus pyogenes* infections induced in mice by intraperitoneal inoculation, without apparent toxic reaction in the animals. Animals first given subtilin 9 hrs. after inoculation were in an advanced stage of the disease but recovered promptly.

Anderson, H. H., Villela, G. G., Hanson, E. L., and Reed, R. K., UC. Some physical and biologic properties of subtilin and other antibiotics. Science 103(2675):419-420. 1946. Partially purified subtilin proved active *in vitro* against *Lactobacillus plantarum*, *Endamoeba histolytica* and its associated bacterium 't', and *Trypanosoma equiperdum*. The concentrations required for antibiotic effect also lowered surface tension appreciably. Intravenous injection of 1 percent solution in mice gave an LD_{50} of 60 ± 3 mg. per kg; subcutaneous injection gave an LD_{50} of 670 ± 30 mg. per kg. When given intragastrically, 5.0 grams/kg. killed. One percent solution instilled into the rabbit's eye was nonirritating.

Chin, Y. C., UC. Biologic properties of subtilin in physiologic saline solution. Fed. Amer. Socs. Expt. Biol. Proc. 6(1):317. 1947. The toxic activity of partially purified subtilin could be reduced without loss of bacteriostatic or amebacidal potency by extracting with 0.9 percent sodium chloride solution. The extract contained the antimicrobial activity.

Anderson, H. H., and Wong, S. C., UC. Antibiotics in experimental tuberculosis. Tuberculology 8(3):77-82. 1946. Partially purified subtilin at 2.5 ppm. prevented growth of virulent strains of *Mycobacterium tuberculosis* in a modified Dubos medium. The partially purified preparations used gave a marked anaphylactic reaction with guinea pigs. Topical application of subtilin was ineffective in treating experimentally induced tuberculous infections of the cornea of rabbits. Daily subcutaneous injections of 6 mg. of subtilin continued over a period of 6 weeks failed to affect the course of experimentally induced tuberculosis in Syrian hamsters. Therapy was initiated 8 days after infection. One particular lot of subtilin was well-tolerated; two others gave marked local tissue toxicity. It was believed that failure of therapy was related to precipitation by physiological concentrations of NaCl. Such concentrations reduced the *in vitro* antibiotic activity of subtilin on *Lactobacillus plantarum.*

Wong, S. C., Hambly, A. S., and Anderson, H. H., UC. Use of modified Dubos and Davis medium for demonstration of antibiotic activity of subtilin against *Mycobacterium tuberculosis*. J. Lab. Clin. Med. 32(7):837-841. 1947. In Dubos medium partially purified subtilin was bacteriostatic at 1/400,000 dilution, and bactericidal only at 1/20,000 or lower dilution. A virulent strain of *M. tuberculosis* was used.

Andersen, H. H. and Chin, Y. C., UC. Antibiotic activity of subtilin and streptomycin in the presence of BAL. Science 106(2765):643-644. 1947. BAL (2,3-dimercaptopropanol) at a dilution of 1/100,000 in Dubos medium increased the bacteriostatic potency of subtilin against 3 pathogenic and 1 nonpathogenic strains of *Mycobacterium tuberculosis* by 2 to 16 times, and of streptomycin to a lesser degree.

Anderson, H. H., UC. New surface active antibiotics. J. Invest. Dermatology 8(1):25-33. 1947. Certain wetting agents, "Tergitol" and "Tween 80", substantially increased the bacteriostatic action of subtilin against *Lactobacillus plantarum*. Sodium lauryl sulfate, on the other hand, greatly reduced the activity of subtilin. Serum albumin (5 percent) or globulin (1 percent) did not repress the activity of subtilin. In Dubos medium with 0.05 percent of Tween 80, partially purified subtilin inhibited growth of *Mycobacterium tuberculosis* for 12 days at 1/400,000 dilution. Subtilin ruptures the cell membrane of *Endamoeba histolytica* *in vitro*.

Chin, Y. C., UC. Effect of some derivatives of subtilin on tubercle bacilli and rabbit leukocytes *in vitro* (abstract). Fed. Amer. Socs. Expt. Biol. Proc. 7(1):211-212. 1948. The tuberculostatic activity of methylated, ethylated, and glycolated derivatives of subtilin against *Mycobacterium tuberculosis* (H37R_V) in Dubos medium was either no different from subtilin or increased 2-fold. The low toxicity toward rabbit leukocytes was unchanged, except for a 3-fold increase for one sample.

Michener, H. D., and Snell, N., WRRL. Antifungal activity of *Bacillus subtilis* cultures (presented before the American Association for the Advancement of Science, Botanical Society of America, at San Diego, Calif., June, 1947). Cultures of subtilin-producing *B. subtilis* possessed antifungal activity which was not exhibited by partially purified subtilin preparations. A single extraction with butanol at pH 2.5 removed most of the activity against Aspergillus niger but not against Rhizopus solani, thus indicating that at least two antifungal substances were present. (Not published).

Goodman, J. J., and Henry, A. W., Dept. of Plant Science, Univ. of Alberta, Edmonton, Canada. Action of subtilin in reducing infection by a seed-borne pathogen. Science 105, 320-321. 1947. Barley seeds exposed simultaneously to a 0.1 percent solution of partially purified subtilin and Xanthomonas translucens cerealis for 24 hours did not yield infected seedlings, but with 0.02 percent subtilin, infection approached that of controls without subtilin. Only partial protection was obtained when the seeds were infected, dried, and subsequently treated with subtilin.

Wilson, R. H., Lewis, J. C., and Humphreys, E. M., Pharmacology Laboratory and WRRL. Subtilin in blood after parenteral administration (abstract). Fed. Amer. Socs. Expt. Biol. Proc. 7(1):266. 1948. Subtilin, subcutaneously, has very low toxicity; 3 gm./kg. of purified subtilin does not kill mice. Because its solubility in physiological saline or serum does not exceed 0.05-0.06 gm./100 ml., subcutaneously administered subtilin precipitates at the site of injection. Intramuscularly administered subtilin likewise is precipitated and absorbed slowly. Amounts up to 100 mg./kg. have been injected into the thigh muscles of rabbits and cup plate assay of periodic blood samples has indicated no more than 2 ppm. of subtilin in whole blood. Examination of the injection site 24 hours after administration revealed heavy deposits of subtilin, and a blood assay 1 month after injection showed a trace of subtilin, indicating that absorption was still in progress.

Intravenous administration produces blood levels of some magnitude. A dose of 10 mg./kg., given as a 1 percent solution in 5 percent glucose in the ear vein of rabbits, gives a blood concentration of approximately 100-200 ppm. in 5 minutes, dropping to 10-30 ppm. in 2 hours and to zero in 24 hours. Greater dosage may kill by interfering with circulation between heart and lungs, presumably by embolism. The intravenous LD_{50} for rabbits is not known accurately; for mice it is about 100 mg./kg. Intravenous infusion (20 mg./kg./hr.) was given a rabbit for 4 hours without producing visible symptoms and led to a final blood level of about 750 ppm.